# 学 位 論 文 の 要 旨

氏名　　片倉　賢紀

学 位 論 文 名　Omega-3 Polyunsaturated Fatty Acids Enhance Neuronal Differentiation in Cultured Rat Neural Stem Cells

発 表 雑 誌 名（巻，初頁～終頁，年）　Stem Cells International（2013 巻, ID490476, 9 頁, 2013 年）

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## 論 文 内 容 の 要 旨

### INTRODUCTION

Polyunsaturated fatty acids (PUFAs), which are critical for the brain development, are classified into omega-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and omega-6 PUFAs, such as arachidonic acid (AA). Many of PUFAs, can not synthesize the required amount from their respective shorter-chain precursors in mammals. Thus, they need to be obtained from dietary sources. Dysregulation of fatty acid and phospholipid metabolism can induce a wide range of psychiatric, neurological, and developmental disorders in adults.

The enhancement of neurogenesis is an important tool to treat brain disorders and has been shown to ameliorate or prevent mental illnesses, cholinergic denervation, and neurodegenerative diseases. We hypothesized that PUFA treatment could prevent or recover these illness and diseases via activation of neurogenesis. Omega-3 PUFAs reportedly enhanced neurogenesis in adult rat brain and AA enhance proliferation and astrogenesis of fetal rat neuronal stem/progenitor cells (NSCs). However, the exact mechanisms of the beneficial effect of PUFAs on neurogenesis have not been elucidated.

Neurogenesis comprises the proliferation and differentiation of NSCs, which involves separate mechanisms; therefore, in the present study, we focused on differentiation of NSCs. Hes1, a repressor type of basic helix-loop-helix (bHLH) transcription factor, is essential for the maintenance and proliferation of NSCs, and their expression maintains the NSCs during embryogenesis. Activator-type bHLH transcription factors such as Hes6, neurogenin, Mash1, and NeuroD enhanced the expression of MAP2, a neuron specific protein, and induced neuronal

differentiation. Cross-talk between these two types of bHLH transcription factors allows some NSCs to undergo differentiation and maintain an NSC state. In the present study, we investigated that the effects of PUFAs on the expression levels of bHLH transcription factors and cell cycle in the NSCs.

## MATERIALS AND METHODS

NSCs were cultured by the neurosphere method. Rat forebrain cortices were isolated on E14.5. The cortices were mechanically disrupted into single cells by repeated pipetting in a serum-free conditioned medium (N2 medium). The dissociated cells were cultured in dishes in N2 medium with basic fibroblast growth factor (bFGF) and heparin in a humidified 5% CO2/95% air incubator at 37°C. Within 3-5 days, the cells grew as free-floating neurospheres that were then collected by centrifugation, mechanically dissociated by pipetting, and passaged. After the second passage neurospheres were mechanically dissociated and plated onto poly-L-ornithine- coated plates containing N2 medium without bFGF and heparin. The cultures were then treated with PUFAs (DHA, EPA, or AA; 1.0 μM) that were dissolved in N2 medium containing 1.0% fatty acid-free bovine serum albumin (BSA) at a final concentration of 0.01%. BSA was used as the vehicle control in this experiment and the culture medium was changed every other day. Cultured cells were fixed and stained with Tuj-1 (an immature neuron marker) or GFAP (a glia marker); then Tuj-1 and GFAP1 positive cells were counted. Total RNA was isolated and real-time polymerase chain reaction PCR was carried out with specific primers. Cell cycle analysis was performed using a 5-bromo-2'-deoxyuridine (BrdU) flow kit following to manufacturer's instruction. Cells were analyzed by fluorescence-activated cell sorting (FACS) using Becton Dickinson FACSCalibur flow cytometer.

## RESULTS AND DISCUSSION

Cell viability was analyzed using methyl thiazol tetrazolium (MTT) assay by treating with 1 μM of DHA, EPA, AA, or 0.01% BSA as a control for 4 days. No change in cell viability was detected in any-PUFA treated NSCs, indicating that 1 μM PUFA was not toxic to NSCs.

On day 4 after differentiation, neuronal cells were identified by staining with anti-Tuj-1 antibody in cells treated with 1 μM of DHA, EPA, or AA, whereas astrocytes were identified by staining with anti-GFAP antibody. Tuj-1-positive cells increased significantly after 4 and 7 d of DHA and EPA treatment, while no difference was observed in AA treated NSCs. These data indicated that omga-3 PUFAs, but not omega-6 PUFAs increased neuronal differentiation of NSCs.

Hes1 mRNA levels were decreased by DHA treatment on day 1and 4, whereas EPA treatment increased Hes1 mRNA expression by 2.5-fold on day 1. AA treatment on day 4

significantly decreased Hes1 mRNA expression. Hes6, an inhibitor of Hes1, was also significantly increased by EPA treatment on day 1, but not on day 4. DHA and EPA treatment significantly increased NeuroD mRNA expression, but AA did not have any effect. The expression levels of Map2 mRNA significantly increased with DHA and EPA treatment, reflecting the change in Hes1, Hes6, and NeuroD expression levels in Tuj-1-positive cells. In the present study, we found that DHA and EPA enhanced neuronal differentiation in cultured NSCs. However, their target effector molecules are different. Our results indicated that EPA itself acts as an enhancer for neuronal differentiation and EPA did not serve solely as a precursor for DHA.

Next, we analyzed BrdU incorporation and total DNA content in differentiating NSCs treated with DHA, EPA, AA, or 0.01% BSA. Proliferating cells (S-phase cells) incorporated BrdU into their DNA and increased the FITC signal intensities. Cell cycle analysis 12 h after DHA and EPA treatment revealed a significant increase in the percentage of G0/G1-phase cells (control 80.8 ± 0.1%; DHA 87.0 ± 0.8%; and EPA 88.5 ± 0.2%) and a significant decrease in the percentage of S-phase cells (control 15.4 ± 0.1%; DHA 8.4 ± 0.5%; and EPA 7.8 ± 0.3%). On the other hand, following AA treatment, 81.2 ± 0.2% of cells were in the G0/G1-phase, 14.6 ± 0.3% in the S-phase, and 2.4 ± 0.2% in the G2/M-phase. To confirm these results, mRNA expression levels of $p21^{cip1}$ and $p27^{kip1}$ (cyclin-dependent kinase inhibitors) were determined in PUFA-treated NSCs. $p21^{cip1}$ mRNA levels in the NSCs treated with DHA and EPA were 4.5- and 2.2-fold higher than in controls, respectively, and $p27^{kip1}$ mRNA levels were 2.5- and 2.3-fold higher than in controls, respectively. These data indicated that omga-3 PUFAs, but not AA induce cell cycle arrest by increasing $p21^{cip1}$ and $p27^{kip1}$ expression levels.

## CONCLUSION

DHA decreased Hes1 expression levels. EPA increased Hes6 expression levels, leading to decreased Hes1 activity. Decreased Hes1 activity increase NeuroD, Map2, $p21^{cip1}$, and $p27^{kip1}$ expression levels. These mechanisms promote neuronal differentiation in NSCs.

論文審査及び最終試験又は学力の確認の結果の要旨

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**論文審査の結果の要旨**

脳の発達・機能維持に重要な多価不飽和脂肪酸（PUFA）のうち魚油に多く含まれるドコサヘキサエン酸（DHA）は、ラットの記憶学習能を促進・改善することが報告されている。申請者らは、この機構の一部に海馬体での神経新生が関与することを既に示唆している。本研究では、PUFAによる神経新生促進機構の一部を解明するため、初代培養神経幹細胞の分化への効果を検討した。ω-3系PUFAに属すDHAおよびエイコサペンタエン酸（EPA）は、神経幹細胞からニューロンへの分化を促進させた。ω-6系PUFAに属すアラキドン酸（AA）は分化に影響しなかった。次いで、神経幹細胞の増殖・分化を制御している塩基性ヘリックスループヘリックス（bHLH）転写因子のmRNA発現量を検討した。DHAは抑制性転写因子Hes1のmRNA発現量を減少させた。EPAはHes1および促進性転写因子Hes6のmRNA発現量を増加させた。DHA、EPAはともに促進性転写因子NeuroDのmRNA発現量を増加させた。DHA、EPAはサイクリン依存性キナーゼ阻害因子p21、p27のmRNA発現量を増加させ、細胞周期におけるG0/G1期の細胞の増加と、S期の細胞の減少を誘導した。AAにはこれら効果はなかった。これら結果は、DHAとEPAはbHLH転写因子の発現量を調節し神経幹細胞のニューロンへの分化を促進させることを示唆する。本論文はω-3系PUFAによる神経幹細胞からニューロンへの分化運命決定機構を明らかにし、脳の発達や修復におけるω-3系PUFAの重要性を示しており、学位に値すると判断した。

**最終試験又は学力の確認の結果の要旨**

申請者はマウス胎仔由来神経幹細胞に対する3種の不飽和脂肪酸が幹細胞の増殖および神経分化に与える影響を解析し、ω-3系のEPAとDHAがNotchシグナルカスケードを抑制することにより神経幹細胞の増殖を抑制し神経細胞へ分化を誘導することで、神経新生を促すことを示した。プレゼンテーションはスピーチ・スライド共にわかりやすく、質疑応答も明快であった。以上より本申請は学位授与に値すると判断した。（主査：松崎有未）

申請者は培養神経幹細胞に異なった多価不飽和脂肪酸を投与することによって、ω-3系多価不飽和脂肪酸が神経幹細胞のニューロンへの分化を促すこと、およびその機構について明確に示した。脳の機能修復を考える上で意義ある基礎的研究であり、関連領域の知識も十分であったので、学位授与に値すると判断した。（副査：安井幸彦）

申請者は培養神経幹細胞を用いてω-3系多価不飽和脂肪酸がニューロンへの分化を促進させることを組織学的、分子生物学的に明らかにした。神経の分化機構を解明しただけでなく、脳の発達や修復に関する重要な知見でもあり、学位授与に値すると判断した。（副査：内尾祐司）

（備考）要旨は、それぞれ４００字程度とする。